Examining the function and mechanism of histone variant H2A.z (HTZ-1) in transcriptional repression.

Undergraduate Research Thesis

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by

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Abstract

Proper gene regulation is vital for development and cell differentiation and one method in which cells regulate genes is via chromatin structure. Histones play an important role in determining chromatin structure and that chromatin structure can influence if a gene is active or inactive. In *C. elegans*, Myb-MuvB/DREAM complex proteins manage inappropriate transcript accumulation by transcriptionally repressing target genes through depositing the histone variant H2A.z/HTZ-1 along the gene body. Here, we studied the *in vivo* characteristics of H2A.z/HTZ-1 mutants and compared them to the known characteristics of Myb-MuvB/DREAM complex mutants. Similar phenotypes suggested that H2A.z/HTZ-1 behaves similarly to and can be classified as a Myb-MuvB/DREAM complex protein. Additionally, we proposed that H2A./HTZ-1 functions in this gene repression pathway to directly condense and close the chromatin state. We also showed that H2A.z/HTZ-1 gene repression function is conserved between organisms by studying characteristics in a different nematode, *C. briggsae*. Studying H2A.z/HTZ-1 associated gene repression is important because H2A.z/HTZ-1 has also been tied to gene activation. A better understanding of exactly how H2A.z/HTZ-1 functions can provide valuable insight into gene regulation.

Introduction

The regulation of chromatin structure is a vital process in multicellular eukaryotic organisms. Every cell in an organism has the same DNA, but yet there are many different cell types. This variation in cells arises because different genes are either active or inactive in each cell type and one major mechanism to control gene expression is chromatin structure (Felsenfeld et al., 1999). Chromatin structure regulates gene expression by managing the accessibility of the transcriptional apparatus to the DNA. An open chromatin state allows for transcription to occur more readily because the transcriptional apparatus can more easily access genes. Oppositely, a close chromatin state restricts this access and thus inhibits transcription (Felsenfeld et al., 1999).

The foundation unit of chromatin is the nucleosome which consists of DNA wrapped around histone proteins. There are four major histones: H2A, H2B, H3, and H4 and four dimers, two H2A-H2B and two H3-H4, along with ~146 bp of DNA that create the nucleosome (McGinty and Tan, 2014). While these canonical histones package DNA after replication, histone variants can replace canonical histones and alter nucleosome structure, nucleosome stability, DNA accessibility, and gene regulation (Weber and Henikoff, 2014). Histone H2A.z/HTZ-1 is a highly conserved histone variant that has many implications in gene regulation. H2A.z/HTZ-1 shares about a 60% sequence similarity with its major histone, H2A. The overall backbone structure between the two is similar, however, the surface of H2A.z includes a metal ion (Suto et al., 2000). This structure supports the idea that H2A.z serves a different function than the major histone H2A.

Besides histone variants, there several protein complexes that function to modulate this chromatin structure, making certain genes or regions either more or less accessible. One protein complex called the Myb-MuvB/DREAM complex participates in the repression of genes. The Myb-MuvB/DREAM complex is a highly conserved complex that manages cellular quiescence and regulates cell cycle genes, cellular differentiation, and germline development (Sadasivam and DeCaprio, 2013). Errors in the Myb-MuvB/DREAM complex are often seen in cancers (Valdes-Mora et al., 2011). The Myb-MuvB/DREAM complex functions through transcriptional repression of target genes by increasing the presence of histone variant H2A.z/HTZ-1 along the gene body of the target genes (Latorre et al., 2015). This relationship connects H2A.z/HTZ-1 to gene repression and cancer development.

A vital component of the Myb-MuvB/DREAM complex is LIN-35/RB. LIN-35/RB operates as a pocket protein that stabilizes the Myb-MuvB/DREAM complex at target genes and in *C. elegans lin-35* null mutants target genes become up-regulated (Goetsch et al., 2017; Latorre et al., 2015). *C. elegans* animals with a nonfunctional LIN-35/RB also have altered H2A.z/HTZ-1 chromatin localization which suggests a link between the two (Latorre et al., 2015). LIN-35/RB has also been seen to primarily associate with genes that have H2A.z/HTZ-1 levels both at the promoter and across the gene body (Latorre et al., 2015). This relationship between LIN-35 and H2A.z/HTZ-1 is understood, however, the exact mechanism of the targeted repression is still unknown.

In addition to this predicted repressive function of H2A.z/HTZ-1, H2A.z/HTZ-1 is more commonly known for its role in gene activation (Stargell et al., 1993). The metal ion on the surface of H2A.z causes destabilization between the H2A.z-H2B dimer and the H3-H4 tetramer (Suto et al., 2000). This weakened interaction between histones when H2Az./HTZ-1 is incorporated into nucleosomes promotes an open chromatin state for increased transcription (Giaimo et al., 2019). Given how the localization of H2A.z/HTZ-1 has been tied to both gene activation and gene repression, it was important to study H2A.z/HTZ-1 associated gene repression to better understand its function and mechanism to differentiate these two processes. This dual function of

H2A.z/HTZ-1 can be seen in cancer, as well, as certain modifications to H2A.z/HTZ-1 have been observed to behave as both an oncogene and tumor suppressor gene (Valdes-Mora et al., 2011).

Since H2A.z/HTZ-1 is better understood for its role in gene activation, we wanted to further confirm the repressive function of H2A.z/HTZ-1. We studied this function by investigating if H2A.z/HTZ-1 functions *in vivo* like other proteins in the Myb-MuvB/DREAM complex in known pathways with Myb-MuvB/DREAM complex associated gene repression, like vulval development and germline-soma distinction. Similar phenotypes would suggest H2A.z/HTZ-1 has similar functions and therefore is a functional component of the Myb-MuvB/DREAM complex. We hypothesized that H2A.z/HTZ-1 would have similar phenotypes to LIN-35/RB and is a functional component Myb-Muvb/DREAM complex.

In this study, it was shown that H2A.z/HTZ-1 does behave like Myb-MuvB/DREAM complex proteins while also having unique characteristics itself by comparing H2A.z/HTZ-1 mutant phenotypes to LIN-35/RB mutant phenotypes. Additionally, by studying the phenotypes of H2A.z/HTZ-1 mutants in a different nematode, *C. briggsae*, it was shown that this gene repression function is conserved amongst organisms.

Materials and Methods

Strains and Cultures

C. elegans and *C. briggsae* strains were cultured and maintained (Brenner, 1974) at 20°C unless otherwise noted. Worms were grown on NGM Agar plates seeded with *E. coli* OP50 as a food source unless otherwise stated. N2(Bristol) was the wild-type (WT) for *C. elegans*. AF16(G16) was the wild-type (WT) for *C. briggsae.* Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Strains used are listed in Supplemental Table 1.

Fluorescent Microscopy

First larval stage animals (L1s) of TH206, CM2778, and CM2779 were studied using fluorescent microscopy to measure the expression level of the PGL-1::GFP transgene, a p-granule protein. Since transgene expression varies from across individual organisms the data was standardized. To standardize the data the histogram/fluorescence levels were compared via a ratio. The histogram/fluorescence levels in the germline precursor p-cell(s) was divided by the histogram/fluorescence levels in a random intestinal cell in individual *C. elegans*. A comparably sized field was used for both cell types. Images were analyzed using the program GIMP2.0.

High Temperature Arrest (HTA) Assays

L4 larvae were placed at either 20°C, 24°C or 27°C overnight and then removed. Eggs were scored (counted). Progeny were scored for L4/adulthood or L1 arrest after two days.

L1 High Temperature Arrest (HTA) Assays

Worms were bleached after being chunked two days prior. Chunking uses a sterilized spatula to move a mass of old agar filled with worms to a fresh plate. L1 worms were placed on NGM-plates and counted. The worms recovered for six hours at room temperature before being moved to the experimental temperature. After two days progeny was scored for L4/adulthood or L1 arrest.

RNAi High Temperature Arrest (HTA) Assays

Empty RNAi vector, MES-4(RNAi), and MES-6(RNAi) were used for this experiment. RNAi bacteria were cultured at 37°C overnight before being placed on RNAi plates with the specific RNAi bacteria. The plates sat at room temperature overnight. L4 larvae were plated at either

20°C, 24°C or 27°C overnight and then removed. Eggs were counted and after two days progeny were scored for L4/adulthood or L1 arrest.

Quantitative RT-PCR Template Creation
C. elegans strains N2, CM2687, MT10430, MT1806, *htz-1(gu243);lin15A(n767), lin-35(n745);lin-15A(n767)* were used for this experiment. Worms were grown on six large plates and bleached once the plates were full. After bleaching, L1 worms were plated on four large plates and recovered at room temperature for six hours and frozen at -80˚C with Trizol. RNA was extracted using the Trizol protocol. RNA was treated with DNase using the Promega/RQ1 protocol. 250 ng of RNA was used in a standard cDNA synthesis protocol. The primers used to amplify the studied germline genes are listed in Supplemental Table 2.

Injection Mix Generation
Injection mixes were developed for a cell-specific chromatin immunoprecipitation (ChIP) experiment. Genetic components for the mixes were amplified from *C. elegans* wild-type DNA. The DNA components were assembled using a Gibson Assembly protocol and the DNA was transformed into pCFJ151 plasmids. These were screened for components using colony PCR. The list of injection mixes created is in Supplemental Table 3. Each injection mix included three components:

25 ng/µl of the unique pCFJ151 plasmid for the cell-specific protein study
25 ng/µl of pCFJ90 as a mCherry selection marker
50 ng/µl of pCFJ601 to increase incorporation of the transgene.

Additionally, one other injection mix was generated using CRISPR-Cas9 to create a GFP::HTZ-1 fusion protein to visualize the expression pattern of H2A.z/HTZ-1. The injection mix is listed below:

I760 - 0.38 µl A2d (repair template), 1.94 µl 9.3 (guide template), 0.46 µl myo-2::GFP, 7.22µl H20

Computational Modeling
The amino acid sequences and structure of HTZ-1(WT) and HTZ-1(GU243) were compared using predictprotein.org and Phyre[2].

Results
H2A.Z/HTZ-1 MUTANTS HAVE CHARACTERISTICS SUGGESTING H2A.Z/HTZ-1 IS A MYB-MUVB/DREAM COMPLEX PROTEIN

In *C. elegans* the multivulva or Muv phenotype can be used to study gene regulation. Vulval development occurs with the activation and signaling of the LIN-3/EGF ligand. This signal induces vulval precursor cells to develop into the vulva (Fig. 1A; Chang and Sternberg,1999). The LIN-3/EGF ligand is often only secreted in a single cell, but when *lin-3/egf* is activated in addition cells a Muv phenotype occurs. There are two main classes of synMuv genes, synMuv A and synMuv B, that help regulate this process. Both synMuv A and synMuv B genes function redundantly to transcriptionally repress *lin-3/egf* (Cui et al., 2006). Due to the redundant function only synMuv A and synMuv B double mutants fail to repress *lin-3/egf* and show a Muv phenotype. LIN-15A is a synMuv A protein and LIN-35 is among the class of synMuv B genes, single mutants of both of these proteins show a wild-type phenotype. However, the *lin-35;lin-15A* exhibits the Muv phenotype (data not shown). Previous work in the Chamberlin Lab created a *htz-1* mutant and a double mutant of *htz-1* and *lin-15A*. If *htz-1* behaved like *lin-35*, a synMuv B gene, in vulva development, then it would suggest functions like a synMuv B gene. The characteristics of these strains were analyzed and it was observed that the single *htz-1* mutant did not exhibit a Muv phenotype, similar to the wild type (Fig. 1B and 1C). However, the *htz-1;lin-15A* double mutants

did exhibit a Muv phenotype (Fig. 1D). Additionally, the number of induced vulval precursor cells in the *htz-1;lin-15A* double mutant was increased compared to the wild-type (Fig. 1E). The Muv phenotype shows improper vulval development and the Muv phenotype in the *htz-1;lin-15A* mutants, but not in the *htz-1* single mutant, suggests H2A.z/HTZ-1 plays a role in the repression of the *lin-3/egf* target gene and *htz-1* could be categorized as a synMuv B gene.

P-granules are germline-specific structures and proteins that can studied to broadly observe overall germline fate (Wang, et al. 2005). Expression of p-granules in somatic cells would suggest mis-regulation of germline genes in certain mutants. To evaluate the ectopic p-granule expression of *htz-1* mutants, an expression pattern also observed in Myb-MuvB/DREAM mutants, a transgenic *C. elegans* strain with an extra-chromosomal array containing PGL-1::GFP was crossed into both *lin-35* and *htz-1* mutant backgrounds. The expression of extra-chromosomal arrays can vary from organism to organism. To standardized the data across multiple organisms, the expression of PGL-1::GFP in the germline precursor cells was compared to a random intestinal cell in individual *C. elegans*. Overall, ectopic p-granule was observed in *htz-1* mutants at 20˚C and 27˚C (Fig. 2). However, the ectopic expression observed in *htz-1* mutants was also significantly different than the expression in the *lin-35* mutants. *htz-1* mutants had a large range of expression levels where some individuals were more wild-type-like, *lin-35*- like, or somewhere in between (Fig. 2B and 2D). This variation could be explained if the mutant H2A.z/HTZ-1 is a hypomorph and cannot be incorporated into the nucleosome as efficiently as the wild-type version. It is possible that when the mutant histone is properly incorporated, a more wild-type-like phenotype is observed. A weaker or decreased incorporation of H2A.z/HTZ-1 could cause a more *lin-35*-like phenotype due to a more open chromatin state and therefore increased expression. Additionally, the p-granule expression in Myb-MuvB/DREAM mutant strains is supposed to be elevated at higher temperatures (Petrella et al., 2011). This characteristic was not observed in either *htz-1* or *lin-35* mutants in these experiments and that could be due to an alternate methodology of the experiment from the initial literature. The ectopic p-granule expression represents overexpression of germline genes and this misexpression is observed in *htz-1* mutants, as well as Myb-MuvB/DREAM complex mutants, showing H2A.z/HTZ-1 functions in gene repression.

To further study germline gene regulation, I investigated if *htz-1* mutants had a high-temperature arrest (HTA) phenotype. The HTA phenotype is caused by mutations in Myb-MuvB/DREAM complex genes that create a more germline-like and open chromatin state in somatic cells (Petrella et al., 2011). This open chromatin state leads to misexpression of germline genes that become lethal to mutant *C. elegans* at higher temperatures (Costello and Petrella, 2019). To investigate this, we ran a HTA assay and the percentage of offspring that developed into L4 or adulthood was observed. Arrested worms were seen mainly at 27˚C and were observed to be smaller and less developed when compared to the wild type (Fig. 3A). It was observed that *htz-1* mutants have a HTA phenotype because they showed a significant survivability reduction at the arrest temperature of 27˚C (Fig. 3B). Additionally, the overall survivability of *htz-1* mutants was significantly lower than *lin-35* mutants with only approximately 50% of the *htz-1* offspring becoming fully developed (Fig. 3B). The HTA phenotype with increased lethality in *htz-1* mutants suggests that H2A.z/HTZ-1 serves similar functions to LIN-35 and Myb-MuvB/DREAM complex proteins for gene repression, specific with germline genes, but also suggests H2A.z/HTZ-1 may have additional functions.

To further confirm that H2A.z/HTZ-1 functioned in the same pathway as LIN-35 and other Myb-MuvB/DREAM complex proteins, we ran a HTA assay with RNAi to knockdown germline chromatin remodeling genes, *mes-4* and *mes-6*. MES-4 (human ortholog NSD2 and NSD3) is a histone methyl transferase that is tied to germ-line determination that promotes an open chromatin

state. MES-6 (human ortholog EED) is predicted to contribute to germline histone methyltransferase activity and should have a similar function to MES-4 (Garvin et al., 1998). By knocking down these genes the chromatin state cannot be initially opened up, therefore the chromatin does not need to be closed to decrease gene expression, which suppresses the HTA phenotype (Petrella et al., 2011). Both *mes-4* and *mes-6* knockdowns saw the suppression of the HTA phenotype in *htz-1s* mutants (Fig. 4A and 4B). The *htz-1s* mutant strain is the *htz-1* mutant with an expected suppressor. The strain still showed a HTA phenotype but had a higher level of survivability than the *htz-1* strain (data not shown). The suppression of the HTA phenotype in H2A.z/HTZ-1 mutants indicates that H2A.z/HTZ-1 is dependent on germline chromatin remodelers, like Myb-MuvB/DREAM complex genes, and functions in the same gene repression pathway.

H2A.Z/HTZ-1 HAS PHENOTYPES SUGGESTING IT FUNCTIONS BY DIRECTLY COMPACTING THE CHROMATIN STATE

Given the difference in the p-granule expression and HTA phenotype between *htz-1* and *lin-35* mutants and the fact, the H2A.z/HTZ-1 had been tied to many other processes we wanted to further study the mechanism of H2A.z/HTZ-1 in gene repression. Since H2A.z/HTZ-1 is a histone variant, we hypothesized that it could directly condense the chromatin state to repress target genes. Therefore, altering the histone could result in more severe phenotypes. One phenotype we tested was if H2A.z/HTZ-1 mutants were more susceptible to the HTA phenotype. *C. elegans* mutants are only susceptible to the HTA phenotype prior to the L1 developmental stage because, by that time period, their chromatin state is most likely already set (Costello and Petrella, 2019). To test this susceptibility, we completed a HTA assay with L1 mutant *C. elegans*. *lin-35* mutants still showed high levels of survivability at the arrest temperature while the *htz-1s* mutants arrested (Fig. 5). The increased period for HTA susceptibility in H2A.z/HTZ-1 mutants suggests the chromatin state remained open longer than usual and it is possible the mutant H2A.z/HTZ-1 was incorporated into the histone, but could not condense it effectively.

If an extended open chromatin state is the cause of the increased HTA susceptibility in H2A.z/HTZ-1 mutants, then one would expect that the expression of germline genes would be higher in H2A.z/HTZ-1 mutants. To test this hypothesis, we are developing a qRT-PCR experiment. Most of the work in this experiment has been in developing the cDNA templates for the PCR reactions. This process requires harvesting RNA from *C. elegans* mutants and generate cDNA from it (Fig. 6A). To date, three of the eighteen necessary templates have been created and confirmed with the other templates still in progress (Fig. 6B and 6C). If this experiment finds the H2A.z/HTZ-1 mutants have increased expression of germline genes than LIN-35 mutants, it would further suggest that the chromatin state is open for a longer period of time.

Furthermore, if H2A.z/HTZ-1 directly condensed the chromatin state, one would also expect it to associate directly on the genes and along the gene body to more extensively regulate the chromatin state. A cell-specific chromatin immunoprecipitation (ChIP) experiment is under development to test this hypothesis. Globally H2A.z/HTZ-1 has been observed to associate along the gene body of repressed genes while LIN-35 associates solely near the transcription start site for targeted repression (Latorre et al., 2014). DNA constructs have been created to study how H2A.z/HTZ-1 associates with *lin-3/egf*, which induces vulval development (Fig. 7; Chang and Sternberg, 1999). Creating these various mutant strains will allow us to directly observe how H2A.z/HTZ-1 interacts with a target gene in different cell types, where the signal is either active or inactive. Additionally, crossing these strains into a *lin-35* mutant background may provide insight into how the association of H2A.z/HTZ-1 is altered, which can provide a mechanistic relationship between H2A.z/HTZ-1 and LIN-35.

H2A.Z/HTZ-1 MUTANT IS NOT PREDICTED TO CAUSE MAJOR STRUCTURAL CHANGES TO THE PROTEIN, BUT MAY INTERFERE WITH NUCLEOSOME STRUCTURE

To better understand the mechanism and function of H2A.z/HTZ-1 associated repression, we compared models of the mutant and the wildtype protein. The *htz-1* mutation caused an Alanine to Valine substitution at amino acid number 66 (Fig. 8A). When modeled with the program Phyre[2], the mutant protein did not show significant changes in the protein structure or secondary structure (Fig. 8B and 8C). To further support the predictions, we utilized predictprotein.org to model the structure of the mutant protein. It predicted a loss of a helix and exposed region, but one predicted helical domain remained (Fig. 8D). Given the conservation of a helical domain, we predicted there are no major structural changes, but more studies should be done to support that claim.

In further predicting the structure and function of the mutant protein, we identified the localization of the mutant in a nucleosome. Since the mutation is in a highly conserved region of the H2A.z/HTZ-1 and H2A main-alpha helix, we were able use images of H2A to predict the effects of the mutation in the nucleosome. Using images created by PDB ID, we identified that the mutated amino acid is associated with histone H2B (Fig. 9). This localization could suggest that the H2A.z/HTZ-1∷H2B association is weaker which could decrease the overall functionality of the histone and its ability to properly incorporate in the nucleosome complex. This predicted structural effect and the phenotypes observed in this study suggest the mutation most likely created a hypomorphic variant.

H2A.Z/HTZ-1 MAY BEHAVE DIFFERENTLY WITH OTHER SYNMUV MUTANTS

The involvement of H2A.z/HTZ-1 in many processes has also led us to further investigate its roles in the vulval development system. Double mutants of *htz-1* and *lin-8, lin-35,* and *lin-37,* respectively are being created (Fig. 10). LIN-8 is a synMuv A protein and if HTZ-1 is a synMuv B protein as predicted, one would expect to see a Muv phenotype. LIN-35 and LIN-37 are synMuv B proteins and therefore we expect to see a normal vulva development when in a double mutant with H2A.z/HTZ-1. However, if that is not the case, it would suggest H2A.z/HTZ-1 can perform functions of other synMuv genes. Using double mutants in the vulva development system provides us a mechanism to test for other possible functions of H2A.z/HTZ-1 in gene repression.

H2A.Z/HTZ-1ASSOCIATED GENE REPRESSION IS CONSERVED IN C. BRIGGSAE

H2A.z/HTZ-1 is a highly conserved histone that is found from yeast to humans (Suto et al., 2000). To determine if the gene repression function of H2A.z/HTZ-1 was also conserved, we tested if H2A.z/HTZ-1 mutants in *C. briggsae*, a sister nematode to *C. elegans*, had a Muv and HTA phenotypes. In *C. briggsae* a single mutation in one synMuv B gene will cause overexpression of the LIN-3/EGF ligand and a Muv phenotype (Chamberlin et al., 2020). *htz-1* mutants in *C. briggsae* were observed to have a Muv phenotype (Fig. 11A and 11B). Additionally, the number of induced vulval precursor cells was also increased in the *htz-1* mutant (Fig. 10C). Misexpression of the LIN-3/EGF ligand in *C. briggsae* H2A.z/HTZ-1 mutant showed H2A.z/HTZ-1 functions as a synMuv B protein is conserved between organisms.

To continue investigating if the repressive function of H2A.z/HTZ-1 is conserved, a HTA assay with *C. briggsae* was run. This experiment proved that *C. briggsae htz-1* mutants do display a HTA phenotype (Fig.12). A background lethality in *C. briggsae htz-1* mutants was also observed (Fig. 12). Additionally, around thirteen percent of *C. briggsae htz-1* were able to escape the HTA

phenotype (Fig. 12). This unique escape, along with slightly higher background survivability in *C. briggsae htz-1* mutants, may suggest a difference in H2A.z/HTZ-1 function between organisms. However, the observation of a HTA phenotype further suggests conservation in the gene repression function of H2A.z/HTZ-1.

Discussion

Gene regulation is a vital process in eukaryotic organisms as it plays a role in development, cell differentiation, and cancer. Currently the histone variant H2A.z/HTZ-1 is most commonly known for its role in gene activation. However, it does also play a major role in transcriptional gene repression, which is less studied. Chromatin structure is a major factor in controlling gene expression, however, the function and mechanism of the histone variant H2A.z/HTZ-1 in effecting that chromatin state is poorly understood. A better understanding of the gene repression function of H2A.z/HTZ-1 will provide deeper insights into gene expression, which would be relevant to treating diseases such as cancer.

In this study, we observed the *in vivo* characteristics of H2A.z/HTZ-1 mutants in *C. elegans* and compared those characteristics to mutants that have known improper gene repression. Additionally, some of those characteristics were observed in *C. briggsae* H2A.z/HTZ-1 mutants. Through a combination of microscopy, survival assays, and other experiments that are still in progress, we have shown that H2A.z/HTZ-1 does have a role in repressing target genes and that this function is conserved between organisms.

H2A.z/HTZ-1 mutants show ectopic p-granule expression and a HTA phenotype that is dependent on germline chromatin remodelers. These phenotypes suggest H2A.z/HTZ-1 functions in the same gene repression pathway as Myb-MuvB/DREAM complex proteins, like LIN-35/RB. This follows the previously studied relationship between H2A.z/HTZ-1 and Myb-MuvB/DREAM complex proteins where Myb-MuvB/DREAM mutants have a decreased level of H2A.z/HTZ-1 deposition on gene bodies and increase gene transcription levels (Latorre et al., 2014). Additionally, since H2A.z/HTZ-1 is a histone and histones directly associate with the DNA, it can be postulated that it serves as the final effector of this pathway and directly modulates the chromatin state itself. This would fit with currently known roles of Myb-MuvB/DREAM complex proteins, which regulate chromatin compaction (Costello and Petrella, 2019).

Interestingly, H2A.z/HTZ-1 was susceptible to a HTA phenotype for an extended developmental period. The HTA phenotype is caused by improper germline gene expression that becomes lethal at higher temperatures (Petrella et al., 2011). HTA is a process that is activated early in development and appears to be irreversible after the critical period before the L1 stage (Costello and Petrella, 2019). This more severe phenotype could be caused by directly altering the compaction of the chromatin instead of solely the regulatory components. The mutant H2A.z/HTZ-1 protein used in this study included an additional one methyl group to its main alpha-helix. This mutation is associated near the H2A.z/HTZ-1::H2B interface which suggests it could interfere with nucleosome formation. The methyl group could inhibit further compaction and thus inhibit the ability to repress the target genes. Additionally, experiments like a Western blot and protein immunoprecipitation experiment could determine the stability of the mutant in nucleosomes, and therefore show if the nucleosome is less stable and more open, like we predict.

Differences in the ectopic p-granule expression and the overall survivability between LIN-35 and H2A.z/HTZ-1 mutant strains could be explained by the H2A.z/HTZ-1 mutation itself. H2A.z/HTZ-1 null mutants are embryonically lethal (Whittle, et al., 2008). Therefore, we expect our mutant H2A.z/HTZ-1 protein is a hypomorph with some maintained function. If this were not the case, then no mutant *C. elegans* would be expected to survive. In terms of p-granule

expression, it is possible that the more LIN-35-like expression in some H2A.z/HTZ-1 mutants was observed when it was poorly incorporated into the nucleosome and a more wild-type-like phenotype was observed when it was properly incorporated. Therefore, the H2A.z/HTZ-1 mutant may decrease the efficiency in which H2A.z/HTZ-1 gets incorporated into the nucleosome. This could also explain the difference survivability where approximately half of all mutants survive since that may be the rate in which H2A.z/HTZ-1 gets properly incorporated in the nucleosome. In this case, improper incorporation would lead to death.

H2A.z/HTZ-1 has been tied to many processes like DNA repair, DNA replication, and gene activation (Giaimo et al., 2019). The role of H2A.z/HTZ-1 in gene activation is very relevant to this study. Future questions should investigate the mechanisms for H2A.z/HTZ-1 associated gene activation and gene repression, as well as separate these two functions. There are many additional avenues to investigate.

Previous work has shown that H2A.z/HTZ-1 levels are higher on DREAM complex gene repression targets versus other genes (Latorre et al., 2011). For this reason, H2A.z/HTZ-1 deposition levels could play a key role in switching between an active or inactive gene state, where more H2A.z/HTZ-1 alters its interaction with other chromatin components thus switching to a repressive function. This interaction could be studied with an *in vitro* system with various levels of H2A.z/HTZ-1 that can be incorporated into a transcription assay. Additionally, H2A.z/HTZ-1, when associated with another histone variant H3.3, further destabilizes the nucleosome thus activating genes (Jin and Felsenfeld, 2007). Therefore, the role of H2A.z/HTZ-1 may be dependent on its surrounding histones. A ChIP assay could determine if there is another histone variant or modification that correlates with H2A.z/HTZ-1 deposition on repressed genes. Also, acetylation of H2A.z/HTZ-1 has been tied to activation while ubiquitination and methylation have been tied to repression (Bruce et al, 2005; Sarcinella et al., 2007; Tsai et al., 2016). Identifying the main modification sites and generating mutant phenotypes could allow further separation of function between these two roles by observing *in vivo* characteristics of those mutants as performed in this project.

Studying H2A.z/HTZ-1 is very relevant to humans and work should continue. HTZ-1/H2A.z has a 90% sequence similarity between C. elegans and humans and is highly conserved amongst eukaryotic organisms (Suto et al., 2000). This project has also shown that H2A.z/HTZ-1 function is conserved with the studies in *C. briggsae* showing similar phenotypes.

Additionally, LIN-35/RB is a known tumor suppressor gene, so tying H2A.z/HTZ-1 with LIN-35/RB function, would suggest that H2A.z/HTZ-1 has a role in cancer that should be further studied. Dysregulation and mutations of the DREAM complex components are frequent in cancers (Valdes-Mora et al., 2011). Modifications to HTZ-1/H2A.z have also already been linked to cancer. An acetylated H2A.z at a transcription start site has been seen to behave like an oncogene and promote gene activation (Valdes-Mora et al., 2011). Therefore, studying the role of HTZ-1/H2A.z as a tumor suppressor with gene repression could provide a better understanding of the different possible mechanisms of HTZ-1/H2A.z, which could expand possible treatments for H2A.z malfunction that is seen in cancers.

Finally, the Muv phenotype in *C. elegans* is caused by overexpression and misregulation of the EGF pathway (Chang and Sternberg, 1999). The Muv phenotype in *C. elegans* is similar to cancers in humans. Errors in the EGF pathway are connected to many cancers including breast, ovarian, and lung cancers (Akhoon et al., 2018). Studying H2A.z/HTZ-1 is not only important to better understanding transcription, gene regulation, and development but will also help tackle the challenges of cancer.

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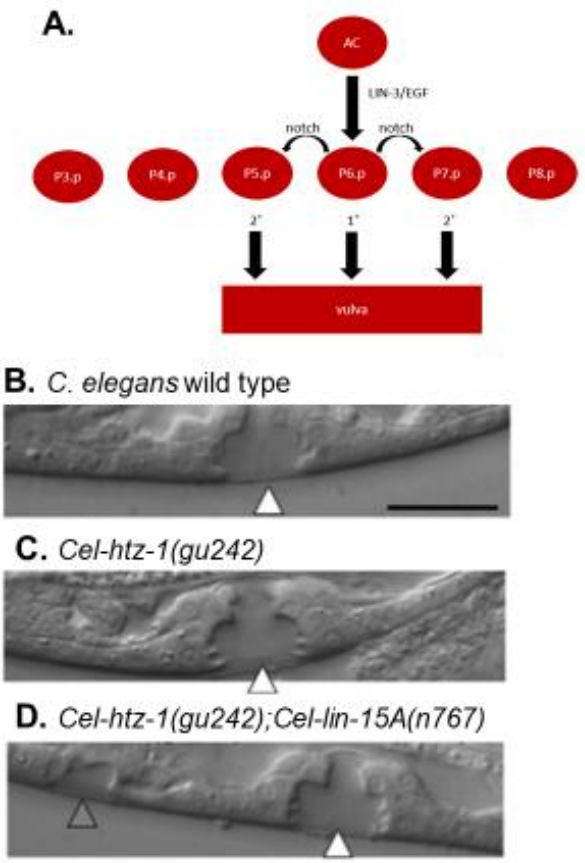
Figures



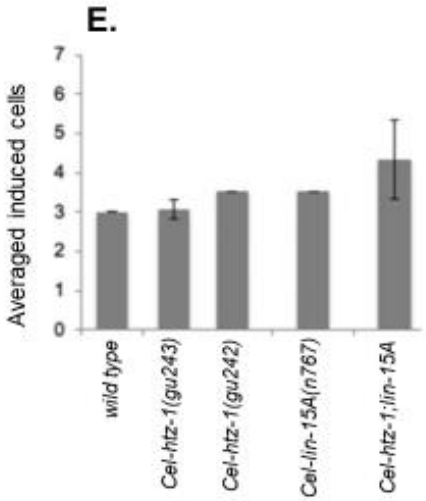


Fig 1. *C. elegans htz-1* mutants exhibit a multivulva (Muv) phenotype with the loss of synMuv A genes. (A) Diagram of vulval development in *C. elegans*. Anchor cell (AC) expresses LIN-3/EGF signal to induce three cells to develop into the vulva during the L4 stage. (B) Wild-type *C. elegans* vulval development (white arrow). (C) Vulval development with *Cel-htz-1* mutant result in little or no change to vulval development. (D) *Cel-htz-1; Cel-lin-15A* double mutants show increased number of induced cells and vulval development (gray arrow). (E) Induced cells in different strains. Error bars correspond to the standard deviation. Scale bar, 20 μm. Figures B-E from Chamberlin et al., (2020).

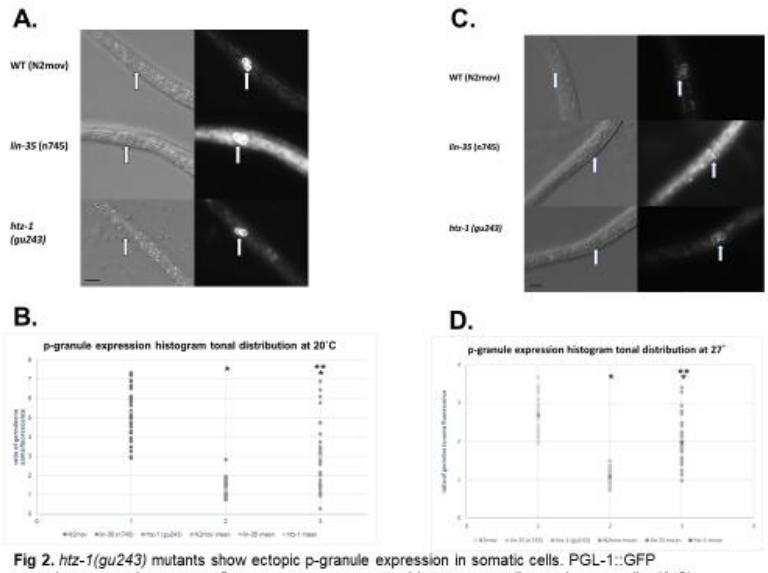


Fig 2. *htz-1(gu243)* mutants show ectopic p-granule expression in somatic cells. PGL-1::GFP extrachromosomal transgene fluorescence was compared between germline and soma cells. (A,C) Expression of PGL-1::GFP was observed in L1 WT, *lin-35, and htz-1* background *C. elegans* at 20˚C and 27˚C, respectively. Wild-type L1s displayed expression in only the two primordial germ cells (arrowheads), while mutants showed ectopic expression. (B, D) PGL-1 expression was compared between the germline and soma cells at 20˚C and 27˚C, respectively. Scale bar, 20 μm. * indicates statistically different from wild type (Z-test, P < 0.01). ** indicates statistically different from *lin-35* (Z-test, P < 0.01).

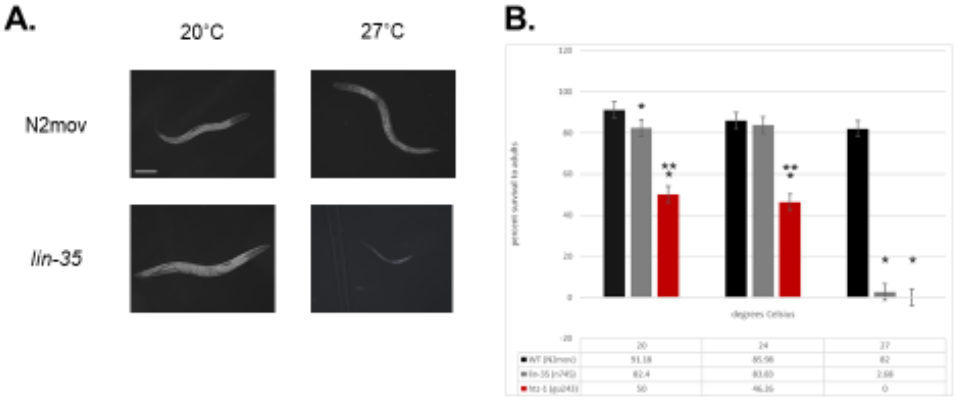


Fig 3. *htz-1* mutants exhibit a high temperature arrest (HTA) phenotype, with increased lethality. (A) Comparison of development result for wildtype and *lin-35* mutants at the arrest phenotype. (B) HTA assay scoring survivability. Scale bar, approx. 100 μm Error bars correspond to 95% confidence interval. * indicates statistically different from N2mov (Z-test, P < 0.05). ** indicates statistically different from *lin-35* (Z-test, P < 0.05).

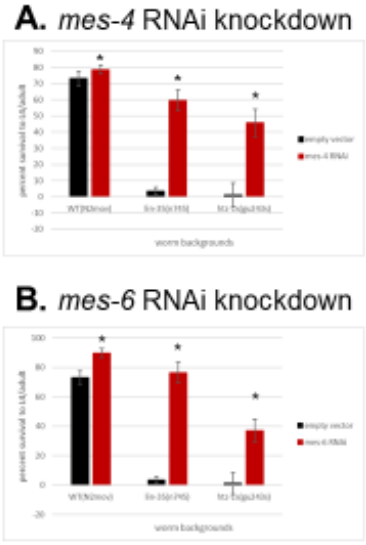


Fig 4. *htz-1s* high temperature arrest (HTA) phenotype is dependent on germline chromatin modifiers. L4 *C. elegans* were transferred to either empty vector or gene-specific RNAi bacteria at 27˚C. Progeny scored for development into L4 or adulthood. (A) HTA assay with *mes-4* knockdown. (B) HTA assay with *mes-6* knockdown. Error bars correspond to 95% confidence interval. * indicates statistically different from empty vector (Z-test, P < 0.05).

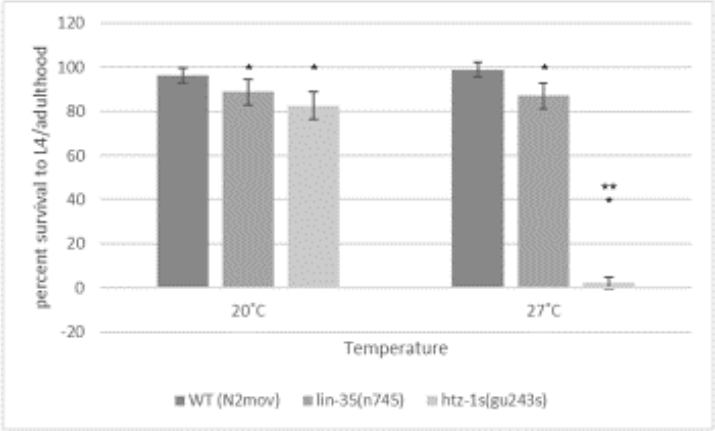


Fig 5. *htz-1s* mutants are susceptible to a high temperature arrest (HTA) phenotype for an extended developmental period. *C. elegans* were synchronized and plated at the L1 stage. Progeny scored for development into L4 or adulthood. Error bars correspond to 95% confidence interval. * indicates statistically different from wildtype (Z-test, P < 0.05). ** indicates statistically different from 20˚C (Z-test, P < 0.05).

A.

1. Grow and bleach adult *C. elegans* on NGM plates
2. Recover L1 *C. elegans* offspring and freeze with Trizol
3. Extract RNA (Trizol protocol)
4. Treat RNA with DNase (RQ1 protocol)
5. Synthesize cDNA from RNA (Chamberlin Lab protocol)
6. Confirm cDNA synthesis with PCR and gel

B.

	qRTpcr				
sample	*growth*	*RNA*	*DNAse*	**cDNA**	*confirm*
N2mov	25-Jul	27-Sep	17-Sep	**24-Feb**	26-Feb
N2mov	5-Sep	10-Sep	3-Oct		
N2mov	25-Sep	17-Oct			
lin-35	31-Jul	2-Aug	3-Oct	**10-Feb**	12-Feb
lin-35	28-Sep	17-Oct			
lin-35					
lin-35	6-Oct				
lin-15A	25-Jul	2-Aug	3-Oct	**10-Feb**	12-Feb
lin-15A	6-Oct	17-Oct			
lin-15A					
lin-15A	11-Sep				
htz-1	29-Feb				
htz-1	7-Mar				
htz-1					
CM2721	11-Sep				
CM2721					
CM2721					
lin35;lin15A			10-Sep		
lin35;lin15A	5-Feb				
lin35;lin15A	5-Sep				

C.

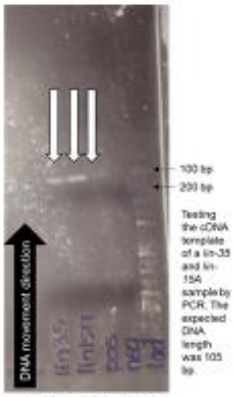
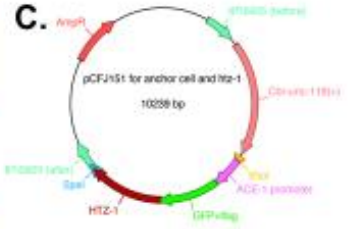


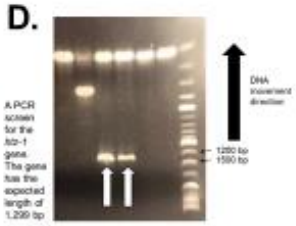
Fig 6. A Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) can be used to determine H2A.z/HTZ-1 regulation of germline genes. (A) Outline of method for qRT-PCR cDNA generation. (B) Progress of cDNA creation. (C) Example PCR results for cDNA confirmation by amplifying *pmp-3*.

1. Create DNA constructs with PCR
2. Transform DNA into plasmid (pCFJ151)
3. Screen colonies to confirm proper transformation
4. Inject DNA construct into *unc-119* mutants
5. Crosslink DNA and H2A.z/HTZ-1
6. Shear DNA-protein complexes
7. Sequence DNA fragments for *lin-3* gene

C.



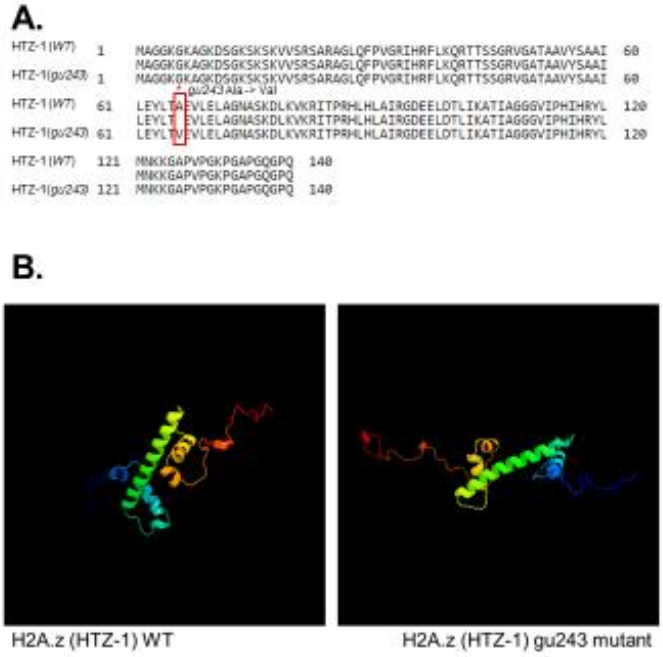
D.



E.

cDNA + 3' UTR	promoters		
	anchor cell (ace-1)	*epidermal cells (dpy-7)*	*muscle cells (myo-3)*
lin-35	✓	✓	i.p.
htz-1	✓	✓	✓

Fig 7. A Chromatin Immunoprecipitation (ChIP) experiment can be used to determine cell-specific H2A.z/HTZ-1 gene interaction. (A) Outline of ChIP protocol. (B) Components of DNA constructs. (C) Generated plasmid for anchor cell and *htz-1* DNA construct. (D) PCR screen for *htz-1* gene to confirm proper transformation. (E) Checklist of completed injection mixes.



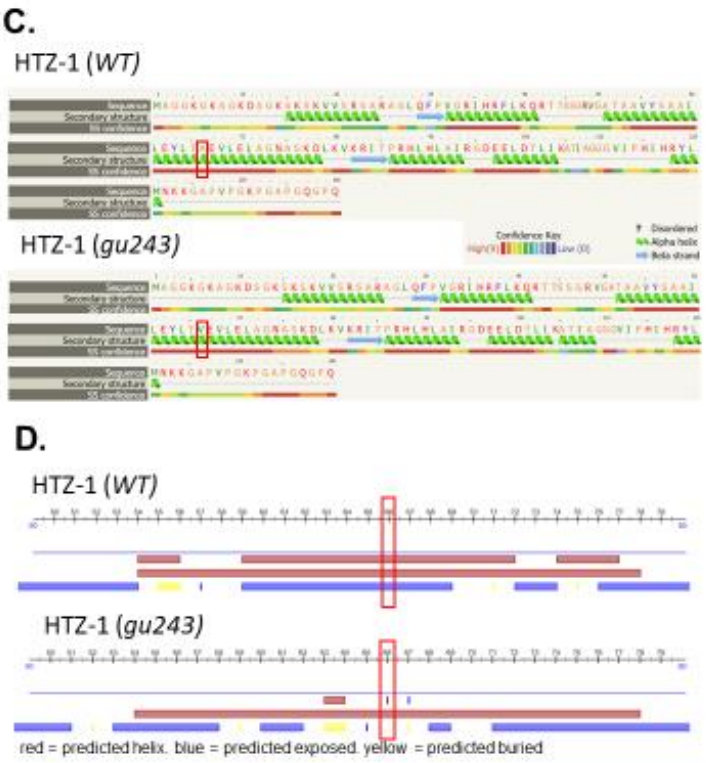


Fig 8. HTZ-1*(gu243)* mutation isn't predicted to cause major structural changes. Substitution location identified with red box (A) Protein sequence comparison for *gu243*. Mutation is Alanine to Valine substitution at amino acid 66. (B) 3D computational models from Phyre[2]. (C) Secondary structure predictions from Phyre[2]. (D) Secondary structure predictions from predictprotein.org.

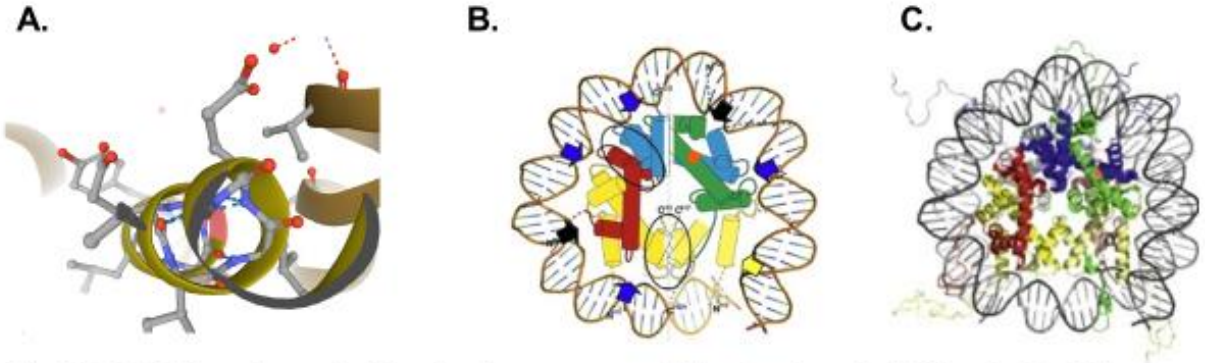


Fig 9. HTZ-1*(gu243)* mutation may interfere with nucleosome structure. (A) Location of mutation in H2A.z main alpha-helix. Figure from Sehnal et al. (2018) (B, C) Predicted location of mutation in nucleosome. Histone H2A is green. H2B is blue. H3 in yellow. H4 is red. Figures from Cutter and Haynes (2015). Mutation highlighted in orange.

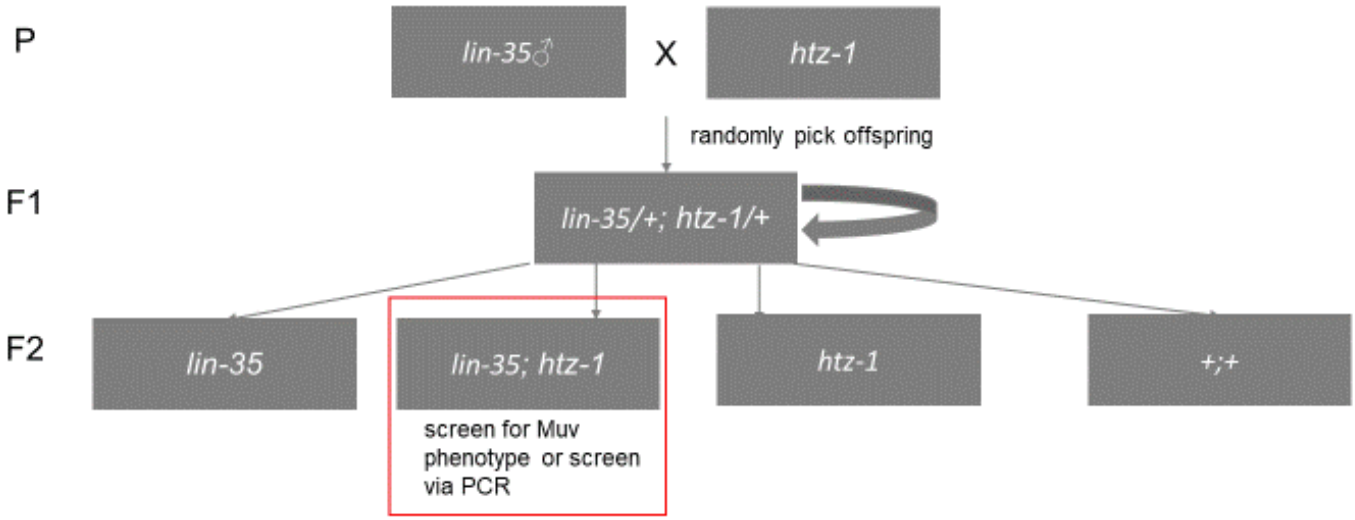


Fig 10. Example *C. Elegans* crossing scheme for *synMuv A;htz-1* double mutants.

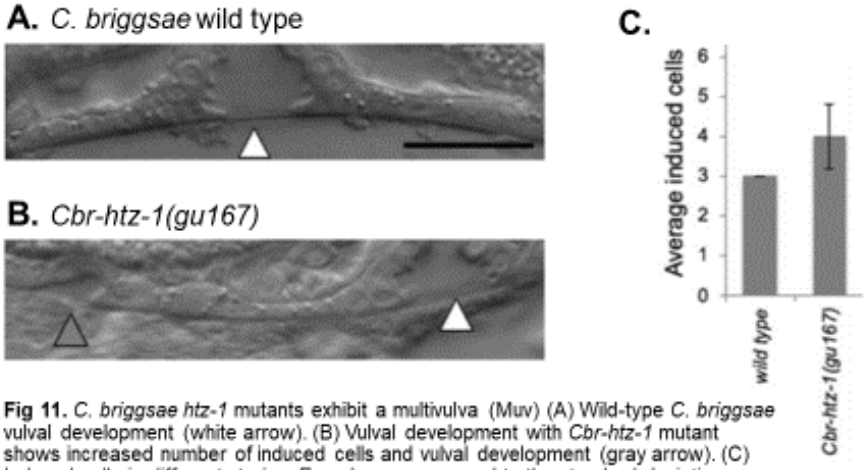


Fig 11. *C. briggsae htz-1* mutants exhibit a multivulva (Muv) (A) Wild-type *C. briggsae* vulval development (white arrow). (B) Vulval development with *Cbr-htz-1* mutant shows increased number of induced cells and vulval development (gray arrow). (C) Induced cells in different strains. Error bars correspond to the standard deviation. Scale bar, 20 µm. Figures from Chamberlin et al, (2020).

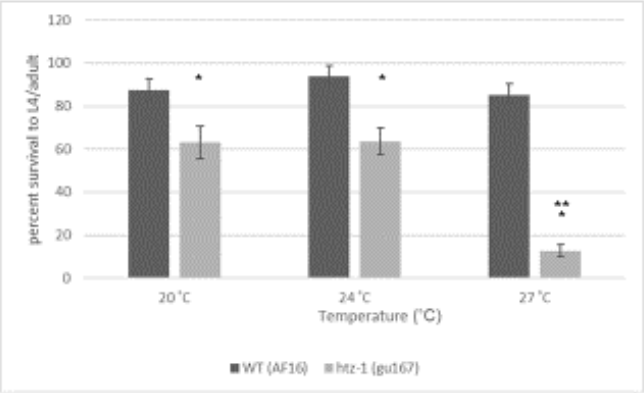


Fig 12. *htz-1* mutants exhibit a high temperature arrest (HTA) phenotype in *C. briggsae*. Progeny scored for development into L4 or adulthood. Error bars correspond to 95% confidence interval. * indicates statistically different from wild type (Z-test, $P < 0.05$). ** indicates statistically different from *gu243* at 20°C (Z-test, $P < 0.05$).

Supplemental Materials

Species	Strain name	genotype	source
C. elegans	N2	*C. elegans* wild type	Chamberlin Lab
C. elegans	CM2686	*Cel-htz-1(gu242)*	Chamberlin Lab
C. elegans	CM2687	*Cel-htz-1(gu243)*	Chamberlin Lab
C. elegans	MT10430	*Cel-lin-35(n745)*	Chamberlin Lab
C. elegans	MT1806	*Cel-lin-15A(n767)*	Chamberlin Lab
C. elegans	MT111	*Cel-lin-8(n111)*	Chamberlin Lab
C. elegans	MT5470	*Cel-lin-37(n758)*	Chamberlin Lab
C. elegans	CM2721	*Cel-htz-1(gu242); Cel-lin-15A(n767)*	Chamberlin Lab
C. elegans	N/A	*Cel-lin35(n745);lin-15A(n767)*	This paper
C. elegans	N/A	*Cel-htz-1(gu243);lin-35(n745)*	In Progress
C. elegans	N/A	*Cel-htz-1(gu243);lin-8(n111)*	In Progress
C. elegans	N/A	*Cel-htz-1(gu243);lin-37(n758)*	In Progress
C. elegans	TH206	*Cel-unc119(ed3);ddEx16 [pgl1::TY1::EGFP::3xFLAG(92C12) + C.br-unc119(+)]*	CGC
C. elegans	CM2778	*Cel-lin-35(n745);unc-119(ed3);ddEx16*	This paper
C. elegans	CM2779	*Cel-htz-1(gu243);unc-119(ed3);ddEx16*	This paper
C. briggsae	AF16	*C. briggsae* wild type	Chamberlin Lab
C. briggsae	CM2031	*Cbr-htz-1(gu167)*	Chamberlin Lab

Table S1. List of worm strains used for this project.

gene	lab oligo number	sequence (5'-3')
pmp-3	5632	ACACTTTCACCGCCCAATGAC
	5633	TCCACATCCACTGTCTCCAGTT
htp-1	6082	GCCCTTGGAAACCATCTACGA
	6083	GGGTCGCTGACAATCCTTGG
htp-3	6084	AGACGTTCCAGTAAACGCACC
	6085	CAACTGGAGGTACAAGCCGAC
rec-1	6086	GACGCCGTGGAGAAATTGC
	6087	TGTAATCACGTCGCCGTCG
rec-8	6088	GGATGCGTTGCCCAACATG
	6089	CGTGGAGTGCATGGTGATTTC
pgl-1	6090	TTCCTCGGCAATCACATCATCC
	6091	AAGTTCCGCAATGGCTCGTC
pgl-3	6092	CGATGGTTGGGATTCTCCGAC
	6093	TGCGGAGAAACCTTGGTGATC
glh-1	6094	TGGAGTGATAGCGAAAGTGCTG
	6095	TTACCACCACCAAACCCAGATC
gpr-2	6096	TGTCTGCTGCCAGATTCATTCC
	6097	AATTGGCACGGAGTCCAGG
pie-1	6098	AGCGCCGTGATTCTCGTTC
	6099	TGGTTGTTGCTGGGTCTCC
mes-2	6100	TATGCGAAGAGACGGCTTGAG
	6101	CGGATCTCTCCTTGGTTTGGAC

Table S2. List of oligo primers used to amplify studied germline genes for qRT-PCR experiment.

Injection Mix Number	Protein	Cell Type	DNA in pCFJ151
I776	HTZ-1	anchor cell	AH3(P*ace-1* :: *htz-1* with utr)
I777	LIN-35	anchor cell	AL11(P*ace-1* ::*lin-35* cDNA::*lin-35* utr)
I778	HTZ-1	hypodermal cells	DH14(7/16)(P*dpy-7* ::*htz-1* with utr)
I779	LIN-35	hypodermal cells	DL23(1)(P*dpy-7* ::*lin-35* cDNA::*lin-35* utr)
I780	HTZ-1	muscle cells	MH9(P*myo-3* :: *htz-1* with utr)

Table S3. List of injection mixes created with DNA inserted in pCFJ151 plasmid for cell-specific ChIP experiment.